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(71) Applicant *(for all designated States except US)*: MOLECULAR SENSORS LIMITED [GB/GB]; P.O. Box 2237, Bradford on Avon, Wiltshire BA15 1YP (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: COBB, Benjamin, David [GB/GB]; Molecular Sensors Limited, P.O. Box 2237, Bradford on Avon, Wiltshire BA15 1YP (GB). CLARKSON, John, Michael [GB/GB]; Molecular Sensors Limited, P.O. Box 2237, Bradford on Avon, Wiltshire BA15 1YP (GB).

(74) Agents: JEHAN, Robert et al.; Williams, Powell & Associates, 4 St. Paul's Churchyard, London EC4M 8AY (GB).

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(57) Abstract

A method for the estimation of a property or parameter of a nucleic acid material, or of a process in which a nucleic acid is modified by a chemical or biochemical reaction, the property or parameter being one to which the electrical conductivity of the nucleic acid material is related, comprises measuring the electrical conductivity of the nucleic acid material, or of a reaction mixturecontaining the material, and estimating from the measurement the property or parameter of the material or process by reference to a predetermined relationship between electrical conductivity and the property or parameter.

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ESTIMATION OF NUCLEIC ACID

This invention relates to the estimation of nucleic acid and more particularly to the estimation of a property or parameter of a nucleic acid or of a process in which a nucleic acid is modified by chemical or biochemical reaction. The invention is especially directed to the determination of concentration (quantification) of nucleic acids in solution and to the determination of molecular weight (sizing) of nucleic acid molecules. The invention is also applicable to the monitoring of reactions in which nucleic acids are modified chemically or biochemically.

Conventionally, DNA concentration is determined spectrophotometrically, a method which relies on the characteristic absorption of ultra-violet light (ca. 260nm) by the nucleotide ring structure of DNA molecules. To determine molecular weight, DNA molecules are normally size-separated by agarose gel electrophoresis and then visualised using the dye ethidium bromide. DNA can also be quantified in agarose gels by comparing with known standards by measuring the fluorescence emitted following excitation by ultra-violet light (ca. 300nm). Both methods have inherent disadvantages. UV spectrophotometers are expensive pieces of equipment requiring the use of costly quartz curvettes and rely on the 'destructive' processing of relatively large sample volumes. The use of ethidium bromide for DNA visualisation and quantification, although a cheaper alternative, is also undesirable due to its extremely toxic and carcinogenic nature.

The present invention comprises a method for the estimation of a property or parameter of a nucleic acid material, or of a process in which a nucleic acid is modified by a chemical or biochemical reaction, said

property or parameter being one to which the electrical conductivity of the nucleic acid material is related, which method comprises measuring the electrical conductivity of the nucleic acid material, or of a reaction mixture containing said material, and estimating from said measurement the property or parameter of the material or process by reference to a predetermined relationship between electrical conductivity and said property or parameter.

The present invention is based on the discovery that there are certain important properties of nucleic acids, the quantitative determination of which is frequently desirable, which can be assessed by measurement of the conductivity of a solution of the nucleic acid or acids. Changes in such properties may be reflected in corresponding changes of electrical conductivity. The concentration of nucleic acid in solution and the molecular weight of a species of nucleic acid are examples of important property which may be determined in accordance with the present invention. Changes of molecular weight occurring in the course of an enzymatic processing of DNA or other nucleic acids may therefore be determined by monitoring changes in electrical conductivity of the solution or reaction mixture.

For the purposes of this invention electrical conductivity may be conveniently measured as the electrical current flowing through a solution of the nucleic acid material.

The present invention is of primary interest for the determination of properties or changes in properties in a single species of nucleic acid. However, absolute purity of the material is not always necessary and the invention is applicable to nucleic acids containing minor amounts or other materials, including other

species of nucleic acid.

The concentration of DNA may be determined by measuring the current/conductivity at a known alternating current frequency. The current recorded at any one fixed frequency has been found to be proportional to the DNA concentration. For example Figure 1 shows how the current/conductivity recorded at 2KHz varies with DNA concentration. This relationship applies not only to a single size of nucleic acid but is true for a range of different sized molecules. In designing apparatus for carrying out the method of this invention a conductivity meter may be readily adapted and calibrated in accordance with the predetermined relationship between current flow and concentration of nucleic acid. In practice it will usually be desirable to calibrate the instrument to deal with homogeneous DNA species but for a range of molecular weights. Thus the sample will normally first be "sized" following which the appropriate nucleic setting for concentration determination will be chosen.

The molecular weight of a DNA species is determined by the response of these molecules to varying frequencies applied across the electrodes. Plotting the current/conductivity recorded (Table 1) as percentage of the maximum current/conductivity response (% response), versus the frequency of the a.c. signal applied between the two electrodes gives characteristic curves which differ for the molecular weights of the molecules concerned (Figure 2).

Table 1

	current (μA)		
frequency	25 mer	puc18	lambda
2.00E+03	6	62	93
4.00E+03	6	63	96
6.00E+03	6	64	98
8.00E+03	6	64	99
1.00E+04	7	64	99
2.00E+04	8	65	100
4.00E+04	11	65	102
6.00E+04	15	66	102
8.00E+04	19	67	102
1.00E+05	24	68	104
2.00E+05	45	78	109
4.00E+05	80	99	120
6.00E+05	98	109	122

For a single DNA species the molecular weight can be determined by firstly calculating the gradient of the response versus frequency curve (over the frequency range 0 - $5x10^5$ Hz). The gradient value can then be compared with a calibration curve (figure 3) of log molecular weight plotted against gradient. For all DNA molecules tested, the gradient varies with molecular weight such that the larger the gradient value, the lower the molecular weight. The basis of this relationship is presumed to be that the mobility of the DNA molecule changes as the frequency changes; as the frequency increases, large molecules are less responsive to changes compared with smaller molecules.

Methods of DNA quantification and molecular weight determination in accordance with the invention circumvent the problems associated with the known methodologies and offer a number of distinct advantages over those conventional methods. Thus, the invention allows rapid and accurate determination of both molecular

weight and concentration; it is more sensitive and accurate, and it requires small sample volumes.

The characterisation of DNA outlined above has been achieved using an a.c. signal, of modulating frequency, between two thin wire platinum electrodes (alternative metals such as copper, stainless steel would also be adequate) through a solution containing DNA. Generally a fixed a.c. signal of between 0 to 10 volts is applied between the two thin wire conductive electrodes. A range of frequencies of this a.c. signal, between 0 and 1MHz, is applied across these electrodes and the corresponding conductivity recorded at each frequency in turn as the current passing through the solution. For example the sample DNA is dissolved in water or TE buffer in a volume of at least 10 μl. For convenience a standard 500 μl plastic tube can be used. Two platinum thin wire electrodes are placed in the solution and connected to a function generator operating at 1 V a.c. A range of frequencies from 0-1 MHz are passed through the solution and the resulting current measured as mA using an ammeter. The DNA concentration is calculated by comparing the current passing through the solution at a frequency of 2 KHz with a standard curve (relating DNA concentration to current at a fixed frequency). Alternative frequencies can also be used.

Specific Example

Confirmation that the molecular weight of DNA molecules in solution can be determined by conductivity methods was achieved by preparing four different DNA solutions in both milli Q water and Tris EDTA (TE) buffer (25 base oligonucleotide, 700 base pair fragment, pUC 18 [=2,690 base pair] and lambda [=50,000 base pair]) and a fifth solution, containing DNA of unknown molecular weight (UN). The conductivity of these solutions was measured in uA over the range of frequencies from 2 x

10^4 Hz to 10^5 Hz. There was no significant difference between milli Q and TE buffer indicating that DNA molecules can be accurately sized in the most common buffer used to store DNA.

To confirm the relationship between molecular weight and conductivity, solutions were prepared in triplicate and tested. Table 2 provides this confirmation and shows that interreplicate variation is slight. Table 3 shows the mean conductivity in uA and also as a % of the maximum conductivity. The main reason for expressing data in terms of % response is that although within experiment variation is low (as shown in Table 1) the fragility of the current probe system causes significant variation between experiments in terms of uA readings. However the trends of conductivity changes (as expressed in % response) is consistent between experiments.

Figure 4 shows the mean % response date from table 3 plotted as a graph. The most obvious difference between the DNA solutions is the gradient of the slopes. When this gradient is calculated from the conductivity at 2 x 10^4 and 4 x 10^5 a near linear relationship is observed between gradient and log molecular weight (figure 5). Thus gradient can be used to calculate molecular weight.

The molecular weight of sample UN found from Fig 5 is about 1047 bps, which corresponds well with estimates made from agarose gel sizing of the same fragment.

Table 2: Conductivity (μA) of DNA solutions over a range of frequencies

replicates 1	2	3	mean (μA)	frequency	size
20	21	22	21	2.00E + 04	
21	22	22	21	4.00E + 04	
22	23	22	22	6.00E + 04	25b
35	35	36	36	8.00E + 04	
54	56	56	55	1.00E + 05	
80	86	82	82	2.00E + 05	
111	115	116	114	4.00E + 05	
32	35	33	33	2.00E + 04	
35	37	38	36	4.00E + 04	
40	40	43	41	6.00E + 04	700 bp
52	54	55	53	8.00E + 04	
63	69	69	67	1.00E + 03	
90	96	93	93	2.00E + 05	
115	118	119	117	4.00E + 05	
51	53	53	52	2.00E + 04	
55	58	55	56	4.00E + 04	
60	64	62	62	6.00E + 04	un
65	71	68	68	8.00E + 04	
69	75	75	73	1.00E + 05	
94	100	100	98	2.00E + 05	
120	130	125	125	4.00E + 05	
68	68	72	70	2.00E + 04	
69	75	70	71	4.00E + 04	
75	80	79	78	6.00E + 04	
76	77	80	77	8.00E + 04	
78	81	82	80	1.00E + 05	2,690 bp
102	104	111	106	2.00E + 05	
135	145	139	139	4.00E + 05	
134	137	138	136	2.00E + 04	
138	142	152	144	4.00E + 04	
139	147	150	145	6.00E + 04	
142	143	153	146	8.00E + 04	
145	154	146	148	1.00E + 03	50,000 bp
151	154	163	156	2.00E + 03	
162	169	169	166	4.00E + 05	

Table 3. Conductivity (as μA and % response) of DNA solutions over a range of frequencies.

25b	700 bp	un	2,690 bp	50,000 bp		
21	33	52	70	136	2.00E + 04	
21	36	56	71	144	4.00E + 04	
22	41	62	78	145	6.00E + 04	μA
36	53	68	77	146	8.00E + 04	
55	67	73	80	148	1.00E + 05	
82	93	98	106	156	2.00E + 05	
114	117	125	139	166	4.00E + 05	
114	117	125	139	166	100%	
18	28	42	50	82	2.00E + 04	
18	31	45	51	87	4.00E + 04	
19	35	50	56	87	6.00E + 04	
32	45	54	55	88	8.00E + 04	% response
48	57	58	58	89	1.00E + 05	
72	79	78	76	94	2.00E + 05	
100	100	100	100	100	4.00E + 05	

The above relationships apply to characterising DNA under static conditions. A further novel application of these relationships is to monitor, in real time, the progress of reactions and processes where DNA is enzymatically modified, for example the polymerase chain reaction (PCR), reverse transcription, DNA-DNA ligation, or where DNA interacts with non-enzymatic proteins, for example DNA-protein interactions. There are a number of advantages which this process facilitates, namely the determination of end-point of the reaction or process, identification of the key stages of the reaction or process, trouble-shooting and automation. For example, PCR is a major molecular biological tool both in academic research and in medical diagnostics, where PCR is used to distinguish individuals who carry specific genetic traits. One of the major bottle-necks encountered in medical diagnostics is the analysis of the PCR products. This usually involves a single pure DNA product which needs to be sized accurately. This is normally carried out by laborious gel electrophoresis.

Measurements of conductivity have also been applied to PCR. A fixed frequency a.c. signal (0 and 1MHz - 0 to 10 volts) may be passed between two thin wire platinum electrodes as above and the current/conductivity measured through the PCR reaction mix as the reaction proceeds. Measurements may be taken at fixed points during the reaction, or constantly. Conductivity fluctuations during the PCR follow distinct patterns which allow real-time assessment of the reaction. Initially there is a drop in conductivity, probably caused by dissociation and denaturation of the genomic template DNA. Successful reactions show a rapid rise in conductivity over the proceeding cycles which reach a plateau which signifies that the end of the reaction has been reached (Figure 4). Unsuccessful reactions show no such increase in conductivity over the proceed-

ing cycles. Experimental details and results obtained are described below.

Real-time monitoring of PCR reactions gave similar results. For reactions which gave poor yields the curve shown in Figure 7 was typical. Initially over the first couple of cycles there is a slight increase in overall conductance (a), this is followed by a decrease in conductance (b) then an increase (c) which levels off to a plateau during the final stages of the reaction (d).

This increase in conductivity during the final cycles of the reaction can be attributed to an increase in the yield of the reaction. The decrease in conductivity during the initial cycles may be accounted for by changes in the concentration of small conducting species such as primers, nucleotides and inorganic ions, whose mobilities contribute to a greater extent than large DNA molecules at the high ac frequencies used. As the concentration of product increases it begins to contribute to the overall conductivity of the solution, thus at high DNA concentrations the conductivity of the reaction mixture increases. When the reaction reaches its end-point the concentration of DNA begins to level off and the conductance of the solution reaches a plateau.

Addition of single reaction components at the end point of the reaction should therefore increase the yield of the reaction further and this would be shown as an increase in the conductivity of the solution. Figure 8 shows how the addition of Taq polymerase (e) increases both the conductivity of the reaction and the overall yield of the reaction. This would suggest that Taq could be limiting. Further experiments at establishing limiting components should include addition of primer, nucleotide and buffer.

It will be recognised from the foregoing that there is provided a method for the estimation of a property or parameter of a nucleic acid material, or of a process in which a nucleic acid is modified by a chemical or biochemical reaction, said property or parameter being one to which the electrical conductivity of the nucleic acid material is related, which method comprises measuring the electrical conductivity of the nucleic acid material, or of a reaction mixture containing said material, and estimating from said measurement the property or parameter of the material or process by reference to a predetermined relationship between electrical conductivity and said property or parameter.

The parameter estimated may be the concentration of a simple species of nucleic acid in a solution thereof. Alternatively, the property estimted my be the molecular weight of a nucleic acid, estimated from the predetermined relationship between molecular weight and the characteristic curve of electrical current/frequency response of a part thereof. The relationship may be between molecular weight and the area under the characteristic curve of frequency response. In a further alternative the property estimated is an overall indicator of molecular weight for a range of nucleic acid species.

The measurement may be carried out with apparatus which has been precalibrated in accordance with the predetermined relationship, which may be both the molecular weight/conductivity relationship and the concentration/conductivity relationship.

If a process parameter is estimated, said parameter may be the extent to which an enzymatic processing of nucleic acid has proceeded, such as a polymerase chain reaction, a reverse transcription, or a nucleic acid ligation.

Appendix

Data acquisition and system design

Measurement of PCR conductivity was achieved by passing a pulsed, 2kHz 1V alternating current through the reaction mixture (Figure 1.0). This was amplified through a variable gain amplifier (1-25) and this signal was logged via a PC. The frequency of signal pulse and data acquisition were also controlled via the PC. An experimental scheme for measuring PCR conductivity is shown in Fig 9.

Digital to analogue control of the switching circuit, and analogue to digital measurements were controlled using a single I/O card (PCL-812PG, Semaphore Systems Limited, London). Initially acquisition of conductivity data was at fixed time intervals, but a significant recent modification (requiring the design of an additional electronic circuit board) has been to regulate acquisition by temperature such that as the temperature reached typical annealing values the rate of acquisition was increased. At high denaturation and extention temperatures acquisition times were increased (Table 4).

Table 4: PCL-812PG I/O card control

Gp	Fn	Parm1	Parm2	Parm3	Remark
01	21	1	2000		Set D/A voltage to 2V (Switch probe on)
02	51			1	Wait 1 second
03	27	229	4		Set internal card gain 4
04	01				Read A/D conductivity probe
05	27	229	0		Set initial card gain 0
06	01				Read A/D conductivity temperature probe
07	21	1	0		Set D/A voltage to 0V (Switch probe off)
08	51			1	Wait 1 second
09	51	$0	4	0.69	If temperature ≤ annealing temperature then 10 If not then 13
10	57			1	Wait 1 second
11	52	1			Goto 01
12	58				End of statement
13				15	Wait 15 seconds
14	00				End

CLAIMS

1. A method for the estimation of a property or parameter of a nucleic acid material, or of a process in which a nucleic acid is modified by a chemical or biochemical reaction, said property or parameter being one to which the electrical conductivity of the nucleic acid material is related, which method comprises measuring the electrical conductivity of the nucleic acid material, or of a reaction mixture containing said material, and estimating from said measurement the property or parameter of the material or process by reference to a predetermined relationship between electrical conductivity and said property or parameter.

2. Method according to claim 1, in which the parameter estimated is the concentration of nucleic acid in a solution thereof.

3. Method according to claim 2, in which the nucleic acid material consists predominantly of a single species of nucleic acid.

4. Method according to claim 1, in which the property estimated is the molecular weight of a nucleic acid.

5. Method according to claim 4, which comprises measuring the conductivity of a solution containing nucleic acid using alternating current over a range of different frequencies.

6. Method according to claim 5, in which the molecular weight of the nucleic acid is estimated from the predetermined relationship between molecular weight and the characteristic curve of electrical current/frequency response or a part thereof.

7. Method according to claim 6, in which the relation-

ship is between molecular weight and the area under the characteristic curve of frequency response.

8. Method according to any of claims 4 to 7, in which the property estimated is a overall indicator of molecular weight for a range of nucleic acid species.

9. Method according to any of the preceding claims in which the measurement is carried out with apparatus which has been precalibrated in accordance with the predetermined relationship.

10. Method according to claim 6, in which the apparatus has been precalibrated with respect to both the molecular weight/conductivity relationship and the concentration/conductivity relationship.

11. Method according to claim 1, in which a process parameter is estimated, said parameter being the extent to which an enzymatic processing of nucleic acid has proceeded.

12. Method according to claim 11, in which the enzymatic reaction is a polymerase chain reaction, a reverse transcription, or a nucleic acid ligation.

13. Method according to any of the preceding claims, in which conductivity is measured by measurement of alternating current flowing through a solution of the nucleic acid material.

14. Method according to any of the preceding claims, in which the nucleic acid is DNA.

15. Apparatus for carrying out a method according to any of the preceding claims, comprising conductivity-measuring means and means for varying the frequency of the applied alternating current.

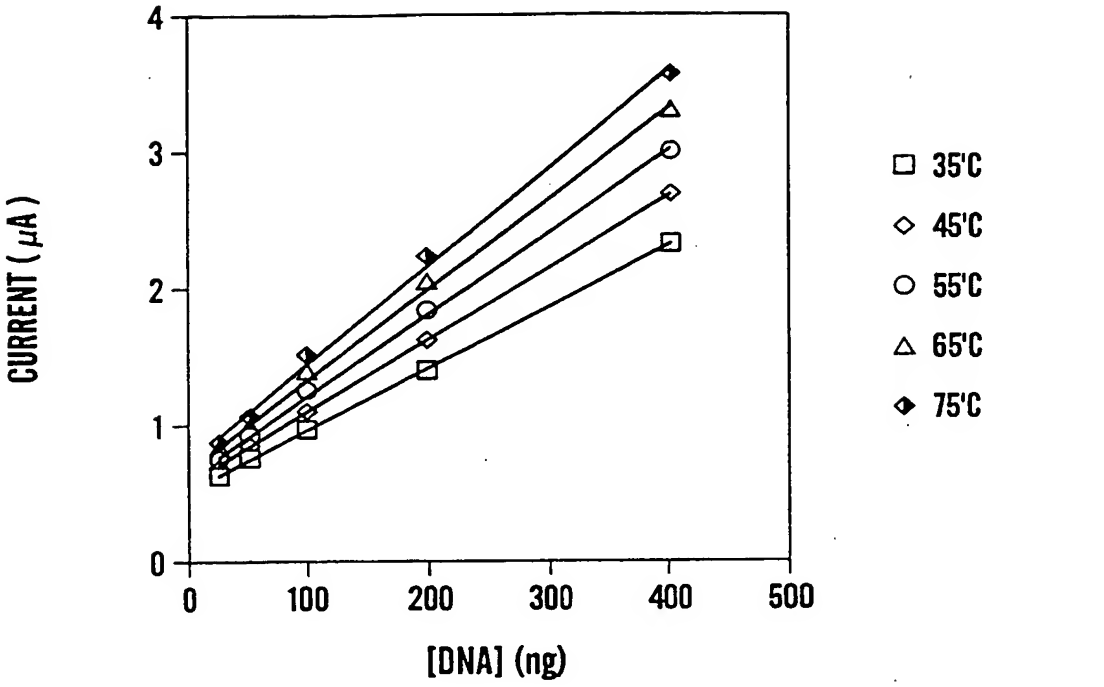

Fig. 1

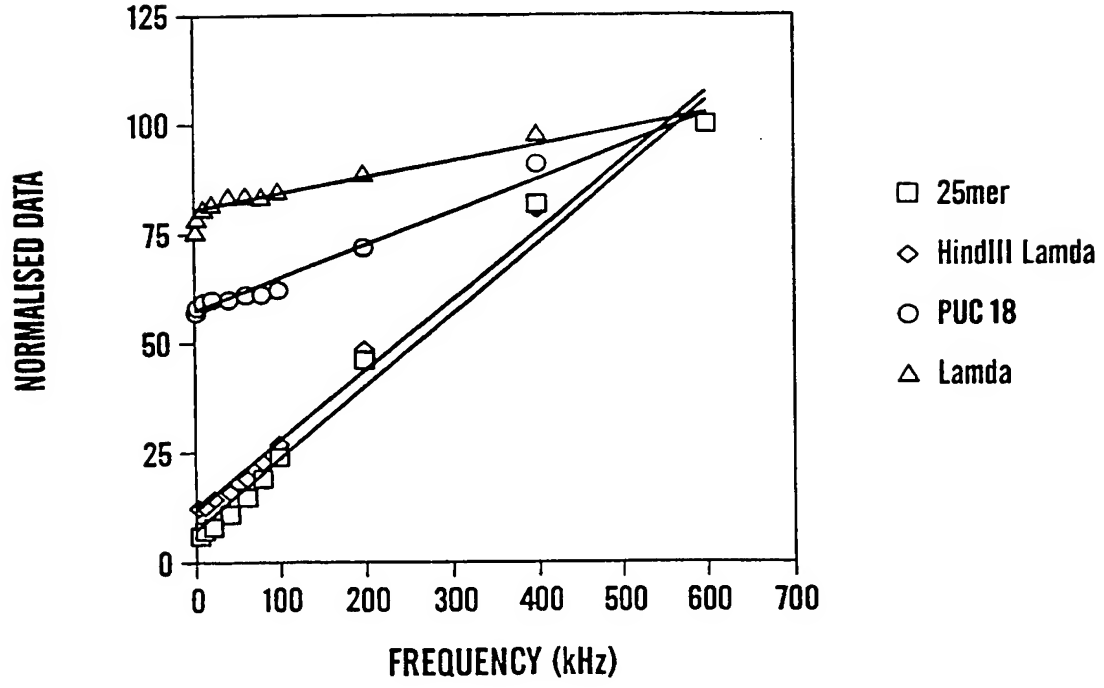

Fig. 2

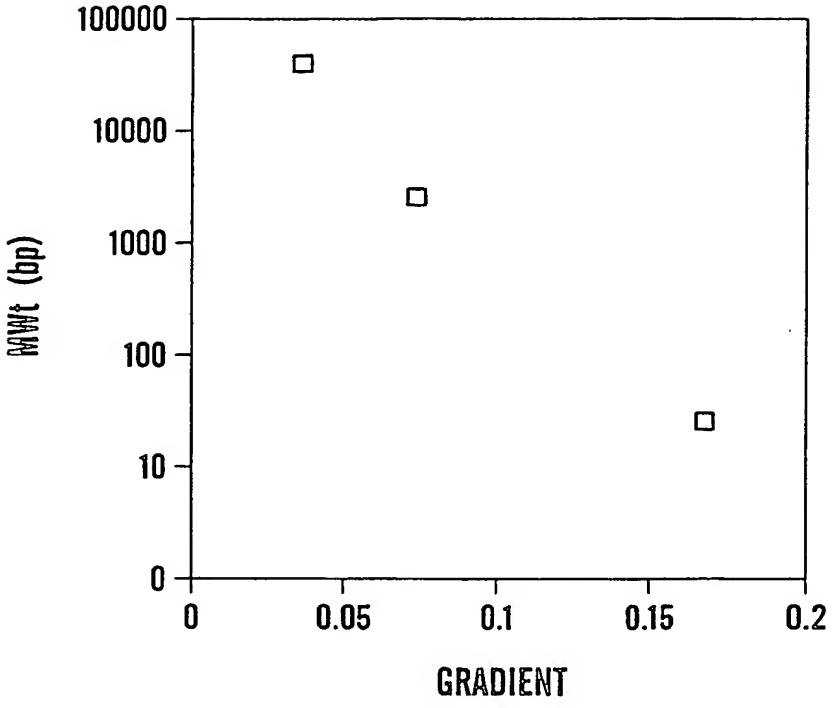

Fig.3

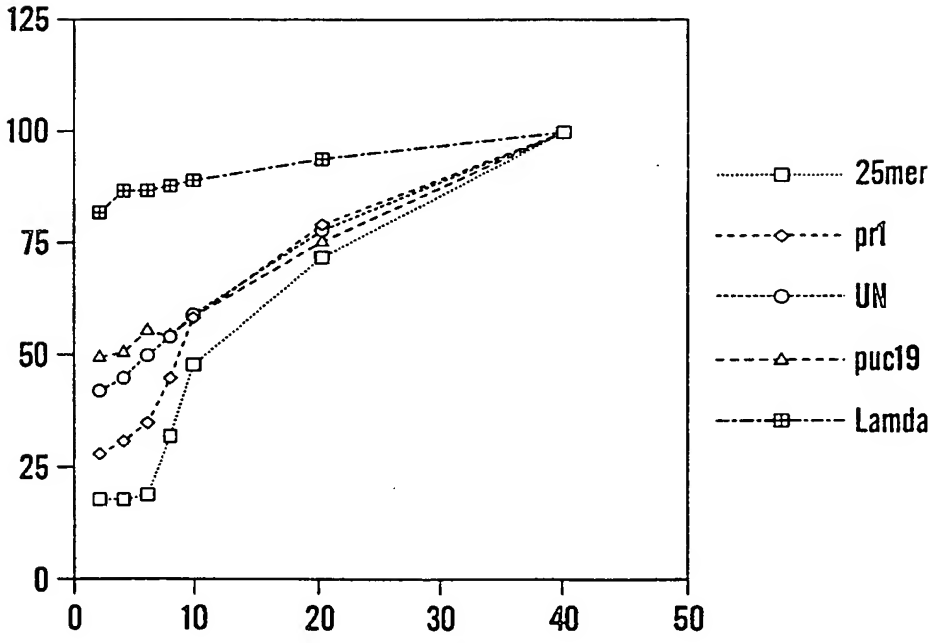

Fig.4

% RESPONSE OF DNA MOLECULES OVER A RANGE OF FREQUENCIES.

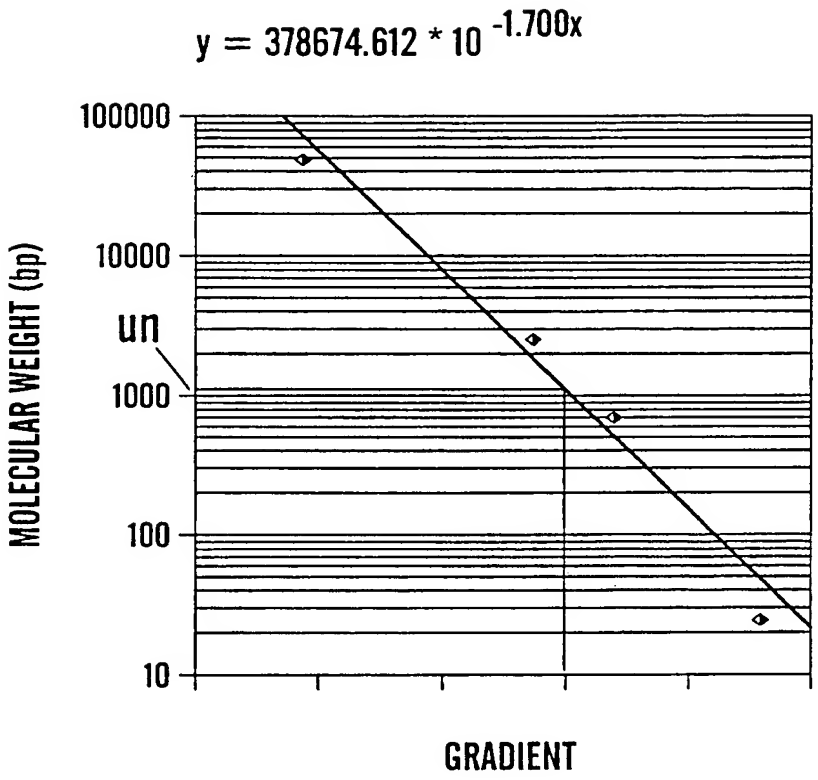

Fig.5

DNA % RESPONSE GRADIENT VERSUS MOLECULAR WEIGHT.

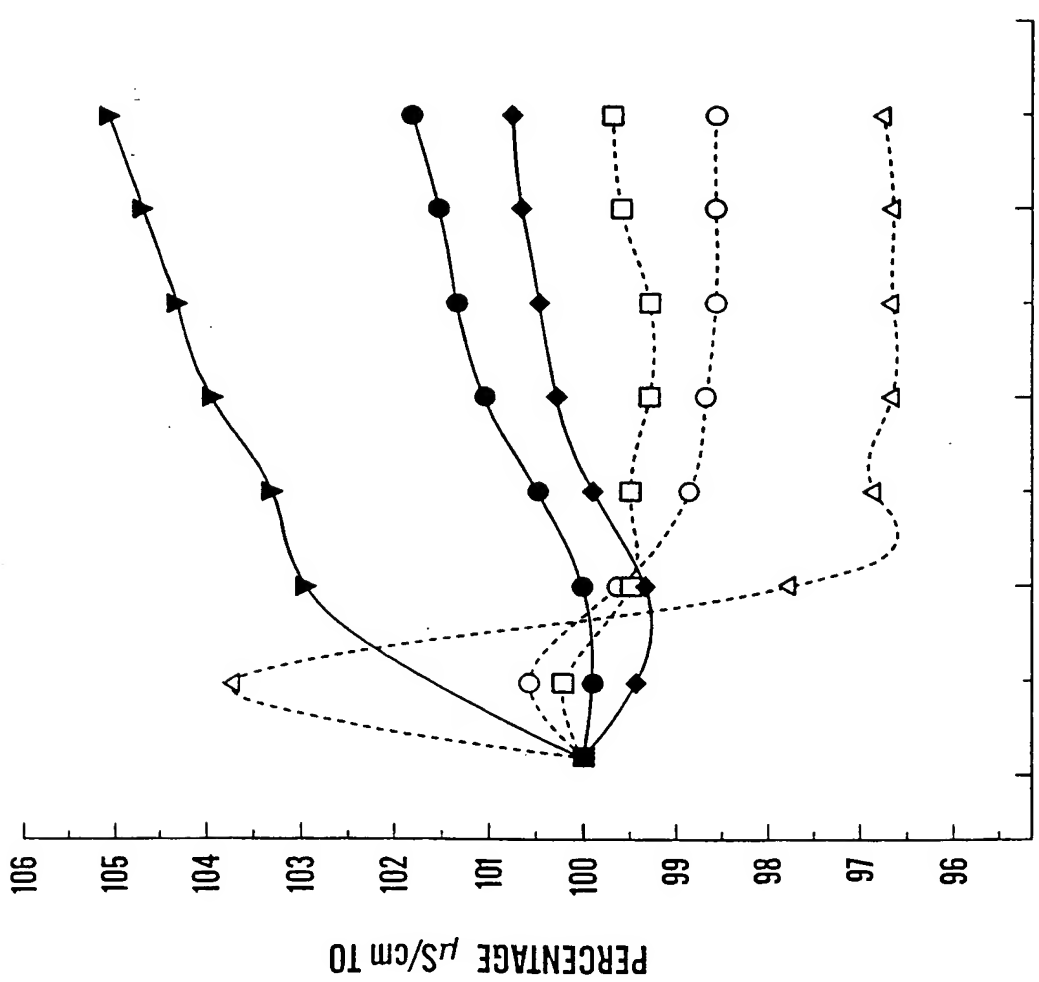

Fig.6 CHANGES IN CONDUCTIVITY DURING THE PCR

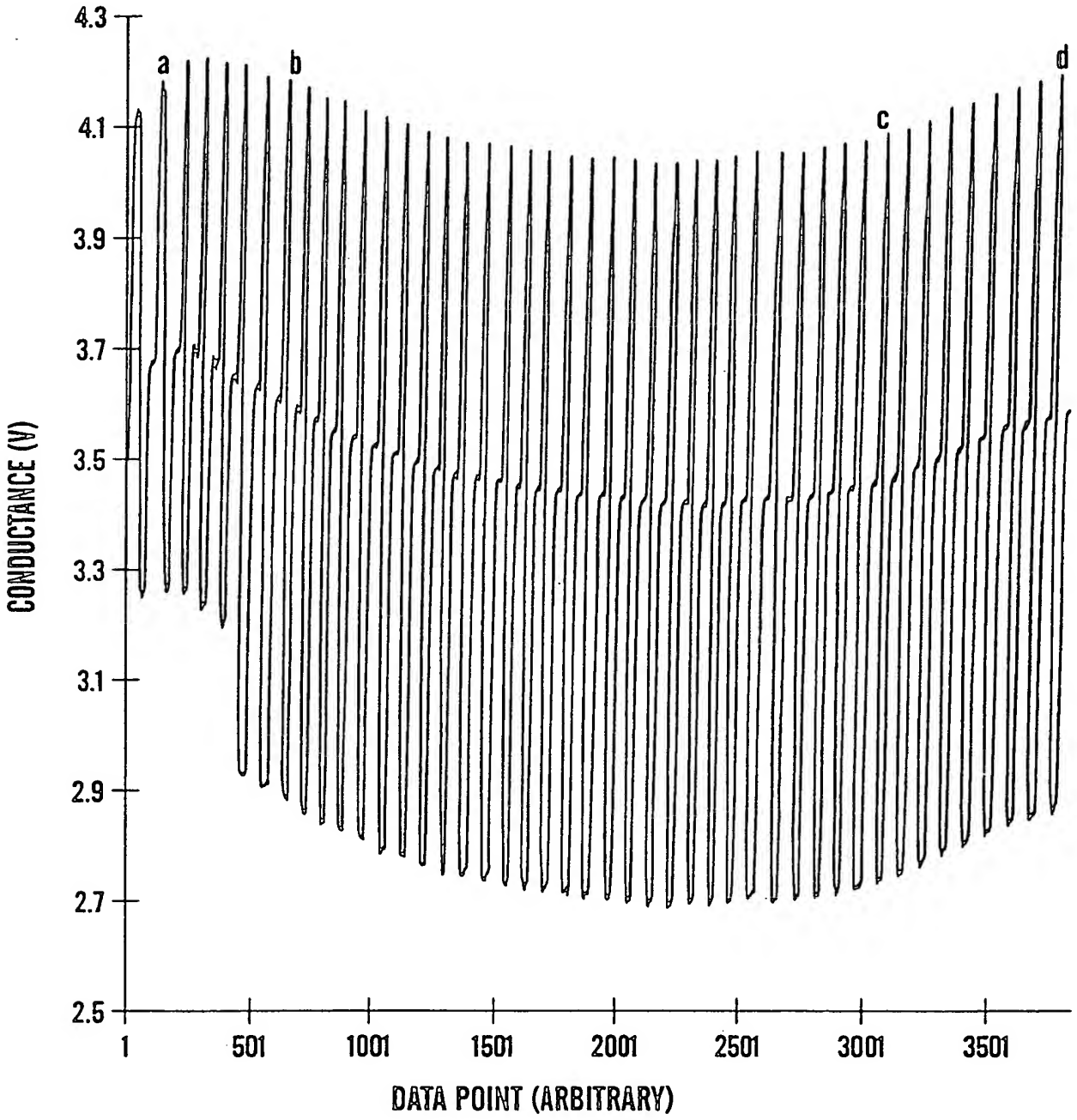

Fig. 7

CHANGES IN CONDUCTANCE DURING AMPLIFICATION OF PR1 FROM PR1-SV50 CLONE.

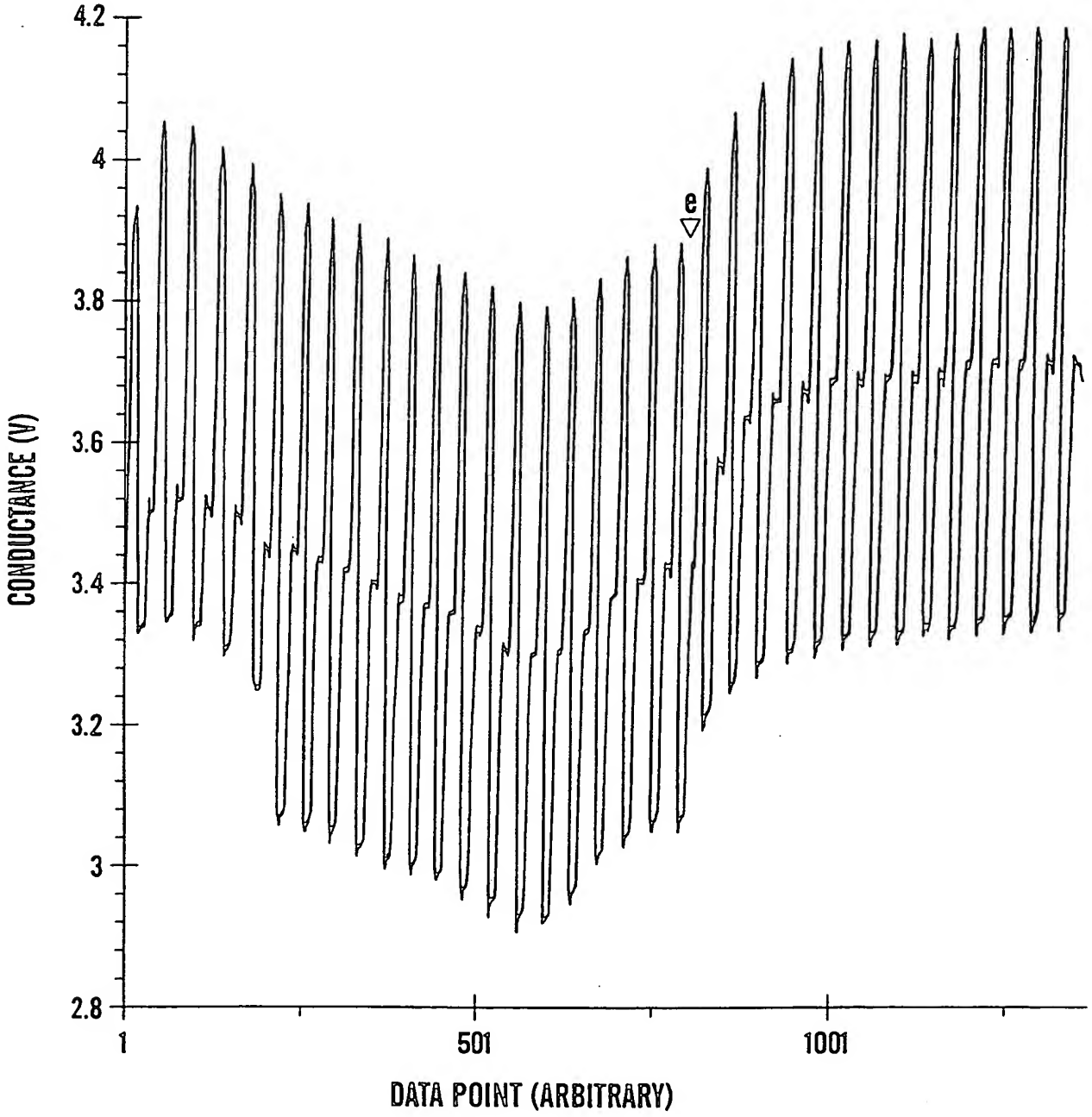

Fig.8

EFFECTS OF ENZYME ADDITION TO AMPLIFICATION OF PR1 FROM PR1-SV50 CLONE.

INTERNATIONAL SEARCH REPORT

Inte..ational Application No
PCT/GB 98/02516

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N27/02 G01N33/487

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 403 451 A (RIVIELLO JOHN M ET AL) 4 April 1995 see the whole document ---	15 1-14
X A	WO 94 02846 A (BRITISH TECH GROUP ;LIU YING (GB); ABEL ERIC WILLIAM (GB); BELCH J) 3 February 1994 see the whole document ---	15 1-14
X	PATENT ABSTRACTS OF JAPAN vol. 010, no. 140 (P-458), 23 May 1986 & JP 60 260838 A (KOUJI ANDOU), 24 December 1985 see abstract --- -/--	15

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